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Original Article

تنوع ژنتیکی و ساختار جمعیتی جدایه های باکتریایی عامل پوسیدگی نرم سیب زمینی در ایران

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چکیده

سابقه و هدف: پوسیدگی نرم از مهمترین بیماری های باکتریایی سیب زمینی در ایران و جهان است. هدف از این مطالعه بررسی تنوع فنوتیپی و ژنوتیپی جدایه های موثر در ایجاد بیماری پوسیدگی نرم در مناطق مهم سیب زمینی کاری ایران بود.

مواد و روش ها: غدد مشکوک به پوسیدگی نرم، در طی عملیات برداشت جمع آوری و مطالعه فنوتیپی جدایه ها بر اساس روش های متداول انجام شد. گوناگونی ژنتیکی نیز توسط روش انگشت نگاری ژنتیکی صورت گرفت. آنالیز کلاستر و بررسی ساختار جمعیت جدایه ها با نرم افزارهای SplitsTree 4.11.3 ، STRUCTURE 2.3.4 و Arlequin 3.11 انجام شد.

یافته ها: مطالعه انجام شده بیانگر تنوع قابل توجه در میان جدایه ها بود. به طوری که برخی از آنها ویژگی های فنوتیپی متفاوتی نسبت به جدایه های معرفی شده در مطالعات قبلی داشتند. جدایه ها در دو گروه فنوتیپی مرتبط با *پکتوباکتریوم واسابیئی* و *پکتوباکتریوم کاروتووروم* زیرگونه *کاروتووروم* قرار گرفتند. انگشت نگاری ژنومی نشان داد که جدایه ها از نظر ژنتیکی ناهمگن هستند و به سه جمعیت ژنتیکی قابل تقسیم بندی می باشند. پنجاه درصد جدایه های گروه فنوتیپی ۱ و ۷۷/۳ درصد جدایه های گروه فنوتیپی ۲ به ترتیب به جمعیت ژنتیکی ۱ و ۳ تعلق داشتند. همبستگی بین پروفایل انگشت نگاری ژنومی، مناطق جغرافیایی و ارقام سیب زمینی مشاهده نشد. اما فاصله ژنتیکی و کمینه و بیشینه جریان ژنی با فاصله جغرافیایی همبستگی نشان داد.

نتیجه گیری: یافته های این مطالعه نشان داد که تجارت غدد بذری یکی از دلایل توسعه بیماری پوسیدگی نرم سیب زمینی به مناطق جغرافیایی هم جوار محسوب می شود.

واژگان کلیدی: ساق سیاه، *پکتوباکتریوم*، پوسیدگی نرم سیب، انگشت نگاری ژنتیکی.

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Original Article

Genetic diversity and population structure of the bacterial agent of potato soft rot in Iran

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Abstract

Background & Objectives: Soft rot is one of the most important bacterial diseases of potato in Iran and the world. The aim of this study was to investigate the phenotypic and genotypic diversity of these agents in the main potato- growing regions in Iran.

Materials & Methods: Samples were collected from tubers that were suspected of soft rot disease during harvest. Phenotypic characteristics were assessed by conventional methods. Genetic diversity was determined using genomic fingerprinting techniques. Cluster analysis and population structure analysis were performed by the SplitsTree 4.11.3, Arlequin 3.11 and the STRUCTURE 2.3.4 software.

Results: The study showed a high level of diversity among isolates so that some of them showed phenotypic characteristics that differed from the isolates described in previous studies. Studied isolates were placed into two major groups of *Pectobacterium wasabiae* and *Pectobacterium carotovorum* subsp. *carotovorum*. Genomic fingerprinting profiles revealed that the isolates are genetically heterogeneous and classified into three genetic populations. 50.0% of the phenotypic group 1 isolate and 77.3% of the phenotypic group 2 isolates belonged to genetic population 1 and 3, respectively. No correlation was observed between genomic fingerprints, geographic areas, and potato cultivars, but the genetic distances and the maximum and minimum gene flow were correlated with geographic distance.

Conclusion: According to the results of this study, seed trade may introduce soft rot disease to new territories in the neighboring provinces.

Keywords: Blackleg, *Pectobacterium,* Potato soft rot, Fingerprinting profiles.
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Introduction

Potato (*Solanum tuberosum* L.) is one of the main staple foods, and the world's most important non-grain food crop. This crop is cultivated worldwide, and as of 2013, it was the fifth main food crop in the world after sugar cane, maize, rice and wheat in terms of total global production (1).

Potato was introduced in Iran during the years 1800-1810 (2). Now Iran is clearly one of the largest potato growers in Central Asia. Potato is planted in nearly all provinces of the country, with total production of 5,164,891 tons across 161,771 hectares of farm lands in 2016 (1). However, there are three major potato growing regions in Iran from which we collected samples: the Alburz Mountains (Ardebil, Tehran), the Zagros Mountains (Hamadan, Kurdistan) and the arid lowlands of southern Iran near the Persian Gulf with some scattered production, especially in the Fars province. The most popular available seed varieties in Iran are Agria, Alpha, Aola, Caesar, Cosima, Desiree, Diamond, Draga, Hermes, Marfona, Picasso, Sante and Savalan.

Potato is affected by some phytobacterial pathogens, including multiple members of the soft rot *Pectobacteriaceae* (SRP) family (formerly soft rot *Enterobacteriaceae*, SRE) (3). In a general sense, the *Pectobacteriaceae* family contains the genera *Pectobacterium*, *Brenneria*, *Dickeya*, *Sodalis* and *Lonsdalea* (4). Of these genera, *Pectobacterium* and *Dickeya* are causing soft rot on potato tubers, meanwhile they have a vast host range and cause disease in half of the angiosperm plant orders (5).

In the appropriate environmental conditions for a pathogen, decaying of the lower stem portion which is known as the black leg disease, is probable. Potato tubers may be infected by soft rot *Pectobacteriaceae* (SRP) *Pectobacterium atrosepticum*, *P. carotovorum* subsp. *carotovorum*, *P. carotovorum* subsp. *brasiliense* , *P. carotovorum* subsp. *odoriferum*, *P. wasabiae*, *P. betavasculorum, Dickeya chrysanthemi*, *D. dianthicola*, *D. dadantii*, *D. zeae* and *D. solani* showing soft rot symptoms in the field or during the storage (6).

In Iran, SRP were isolated from different hosts, including potato, cabbage, sugar beet, pepper, carrot, lettuce, rice, onion, cucumber, turnip, tomato, some ornamental plants and wild flowers (7-13). Most of these studies revealed that there is heterogeneity among SRP isolates in Iran.

In addition, methods for investigating the variability within and among the causing agents of potato bacterial soft rot are examined, including Rep-PCR genomic fingerprinting (14,15), PCR-RFLP of 16S or 16S-23S rDNA intergenic spacer (16), PCR-RFLP of recA gene fragments (12) and AFLP fingerprinting (17).

Repetitive sequence-based PCR (REP-PCR) is a typing method that generates DNA fingerprinting which in turn discriminates bacterial strains (6).

Rep-PCR fingerprinting has been developed to target the repetitive sequences dispersed throughout the bacterial genomes, namely Repetitive Extragenic Palindromic elements (REP), Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR) and BOX elements (BOX-PCR) (18). This method allows phylogenetic classification of SRPs from genus

down to strain level (19).

The purpose of the present study was identification of *Pectobacterium* species causing potato soft rot, as well as investigation of phenotypic and genotypic diversity of these pathogens in major potato growing areas of Iran.

Materials and Methods

Strain collection

The study region was situated in the five important potato growing provinces, namely, Kurdistan, Hamedan, Ardebil, Tehran and Fars (Figure 1), which were the largest potato producing areas in Iran. Samples were collected from suspected tubers during harvest. Information on geographical locations and host cultivars are listed in Table 1. *Pectobacterium carotovorum* subsp. *carotovorum* SCRI214 and *Pectobacterium carotovorum* subsp. *atrosepticum* SCRI1043 were obtained from

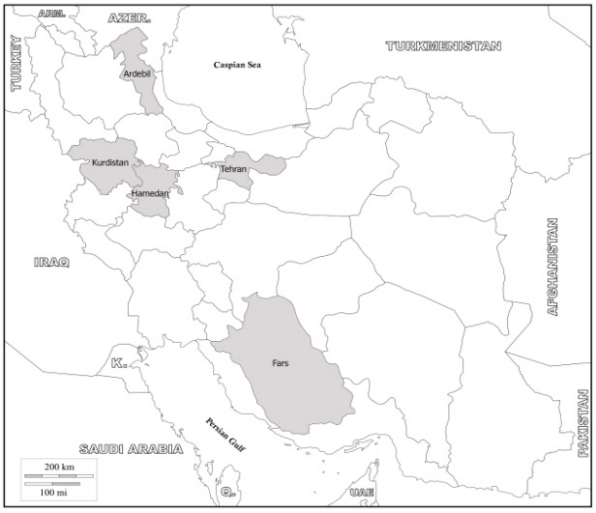

Figure 1: Map of Iran showing the five main collection sites of soft rot *Pectobacterias* associated with potato blackleg strains. Ardebil, Kurdistan, Hamedan, Tehran and Fars provinces are shown in grey; more details of the collection sites are provided in table 1.

Table 1: Information on geographical locations, host variety, and date of sample collection

Main Region	samples collected	Province	Territory	Variety	Sampling Date
1	# 48	Ardebil	Tupraghlu	Agria	Sep., 2006
			Piragum	Agria, Caesar	
			Nyar	Agria	
			Nojeh Deh	Agria, Satina Draga,	
			Zarnas	Agria	
			Aghabagher	Caesar	
			Tazeh-Kand	Agria	
	# 25	Tehran	Damavand, Bidak	Agria	Oct., 2006
			Damavand, Absard	Agria, Marfona	
			Damavand, Homand	Agria	
			Firouzkuh, Gaduk	Agria	
			Dehgulan, Cheragh Abad	Agria	
			Dehgulan, Mubarak Abad	Agria	
			Dehgulan, Karim Abad	Agria	
			Dehgulan, Amir Abad	Agria	
2	#51	Kurdistan	Dehgulan	Agria	Agu.-Sep. 2005
			Dehgulan, Chaghmagh Dare	Agria	
			Gurveh, Veynesar	Agria	
			Dehgulan, Kuche Abad	Agria	
			Dehgulan, Gurv Chai	Agria	
			Gurveh, Shekuh Abad	Agria	
			Dehgulan, Sarab	Agria	
	#37	Hamedan	Razan, Damaq	Agria	Sep., 2006
			Razan, Vafs	Agria, Marfona	
			Razan, Kahriz Boghazi	Agria	
			Razan, Khorvande	Marfona	
			Kabudarahang, Qaragol	Marfona	
			Kabudarahang, Amir Abad	Arinda, Agria, Satina, Marfona	
3	#11	Fars	Siakh Darengan	Agria	Oct., 2006
			Khan Khamis Olia	Agria, Marfona	

Scottish Crop Research Institute and were used as standard isolates for comparative study.

Growth of bacteria

All the strains were grown and isolated on Eosin Methylene Blue Agar (EMB) medium at 28°C. Individual colonies were selected and subcultured on Nutrient Agar in order to obtain pure cultures. For long-term storage, strains were cryopreserved at -70°C in 15% glycerol and 10% skim milk, and were kept in the phytobacteriology collection of Islamic Azad University, Science and Research Branch, Tehran, Iran. All tests used fresh culture of bacteria which was grown on the NA surface and kept in an incubator for 48h at 27-28°C.

Pathogenicity test

To confirm their pathogenicity, all the strains were test-inoculated onto the potato tubers (*Solanum tuberosum* L. cv. *Agria*) by bacterial suspension concentration of 1×10^6 cfu/ml. After inoculation, injected site of tubers was covered by Parafilm and tubers were placed in incubator at 25°C for 6 days. As a negative control, potato tubers were treated with sterile distilled water as well (20).

Phenotypic studies

Biochemical and physiological characteristic of the isolates that are shown in table 2, were assessed by using previously described methods (21-23). All tests were launched at 27°C, repeated three times to ensure the results were consistent, and were read after two to twenty days based on reference literature.

DNA preparation

Total genomic DNA was extracted from isolates using a simple lysis of cells. Colonies were picked directly from plates, were suspended in 1000 µl Sterile Distilled Water (SWD) and were added with 25 µl of NaOH 5%. They were then heated in warmed bath at 95°C for 10 min.

Genomic fingerprinting

Genetic diversity of the isolates was determined using rep-PCR fingerprinting technique. Three sets of PCR primers were performed to amplify endogenous interspersed repetitive sequences, including enterobacterial repetitive intergenic consensus sequences. These include ERIC1, ERIC2 and BOXA1R elements, which are collectively known as rep-PCR (18, 24).

Thermocycle programs and oligonucleotide primers used to amplify the ERIC1, ERIC2 and BOXA1R DNA elements were based on methods that were described by Versalovic *et al.* (18). Primer sequences corresponding to ERIC – ERIC1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC2 (5′AAGTAAGTGACTGGGGTGAGCG-3′) and BOXA1R (5′-AAGTAAGTGACTGGGGTGAGCG-3′) were obtained from Roche (Penzberg, Germany).

Each 25 µl reaction mixture contained 2 U Taq DNA polymerase, 2.5 µl 10 × PCR buffer, 1.25 mM of each deoxynucleoside triphosphates, 50 pmol of each primer, and 100 ng template DNA. Temperature cycling was controlled in a Bio-Rad cycler programmed as follows: one cycle of denaturation at 95°C for 7 min, 30

cycles of denaturation at 90°C for 30 seconds, annealing at 52°C for 1 min with BOX1AR and ERIC primers, extension at 65°C for 8 min with a single final extension cycle at 65°C for 16 min and a final soak at -20 °C. The sizes of PCR products were verified by DNA Ladder (SM #0331; Thermo Scientific, USA).

Estimation of the PCR-amplified products' sizes was done by electrophoresis in 1.5% agarose gels in TBE buffer (25).

Data analysis

To figure out the phenotypic diversity between isolates, a binary data matrix containing the positive (1) or negative (0) response of each test for each isolate was generated using MS EXCEL 2013 software. A dendrogram was constructed as well using Jaccard's coefficient and UPGMA method in NTSYS-pc. Similarly, to determine the genotypic variation of the isolates, for each sample, ERIC1, ERIC2 and BOX bands were scored as 1 (present) or 0 (absent). These binary data were used to build a rectangular matrix in MS EXCEL 2013.

Cluster analysis and population structure were performed by the SplitsTree 4.11.3 software (26) and the STRUCTURE software revised version 2.3.4 (27).

The program SplitsTree 4.11.3 was used to visualize the relationships among the Iranian SRP isolates, which were displayed as a phylogenetic network. Distance analysis was conducted by the Neighbor-Net algorithms (28) which are implemented by the mentioned software.

Support values at each node in the mentioned network were estimated by running 1000 bootstrap replicates. Also we used the Bayesian analysis based method in order to confirm the results of the cluster analysis.

Using the Structure 2.3.4 software, the K1 to K8 assumptions were implemented (K representing the number of groups), and then based on the ΔK values, the most correct grouping was identified. The genetic structures of the Iranian SRP populations at the different sampling sites were compared based on an FST test using Arlequin software version 3.11 (http://cmpg.unibe.ch/software/arlequin3/) (29). An analysis of molecular variance (AMOVA) was performed to partition the genetic diversity among different regions and among populations within regions (30). Also, Nei's genetic distances were computed between the Iranian isolates populations and their significance assessed by bootstrap (31).

Results

Strain collection

Eighty five bacterial strains in total were isolated and purified from 172 naturally infected potato tuber samples. These samples were gathered from 91 different potato farms situated in 30 territories of the five major potato producing regions within Iran (Figure 1, Table 1).

All of the strains grown on Eosin methylene blue agar (EMB) medium formed circular, slightly convex and entire colonies with regular edges and metallic color. On the King's B medium, isolates had round and cream colonies.

Pathogenicity test

Typical soft rot symptoms appeared at the inoculation site on potato tubers of cv. Agria

after 6 days. For a negative control, no soft rot symptoms were observed on potato tubers treated with sterile distilled water.

Phenotypic studies

All isolates with positive pectinolytic activity were Gram-negative, rod-shaped, motile by peritrichous flagella, catalase positive, facultative anaerobic and non-sensitive to erythromycin (15 µg/disc). All of them reduced nitrates to nitrites and caused soft rot on potato slices within 24h at 26°C temperature. Negative

Table 2: Basic characteristics of studied Iranian strains compared with *Pectobacterium* species, subspecies and *Dicheya chrysanthemi*[†]

Characteristics	Present isolates (*n=85*)	*P. c.* subsp. *carotovorum* [†]	*P. atrosepticum*[†]	*P. betavasculatum*[†]	*P. wasabiae*[†]	*Dicheya chrysanthemi*[†]
Gram positive reaction	0[*]	-	-	-	-	-
Motility	100	+	+	+	+	+
Fluorescent pigment on King's B medium	0	-	-	-	-	-
Catalase reaction	100	+	+	+	+	+
Facultatively anaerobic growth	100	+	+	+	+	+
Nitrate reduction	100	+	+	+	+	+
Gelatin liquefaction	91.56	+	-	V	+	+
Lecithinase	1.6	-	-	-	-	+
Oxidase	0	-	-	-	-	-
Catalase	100	+	+	+	+	+
Potato soft rot	100	+	+	+	+	+
Reducing substances from sucrose	11.5	-	+	+	-	+
Blue pigment on YDCA	0	-	-	-	-	+
Methyl Red	60	+	+	-	+	+
Indole production	0	-	-	-	-	+
Acetoin production	32.4	+	V	+	-	+
Urease production	0	-	-	-	-	-
Phenylalanine deaminase	0	-	-	-	-	-
H_2S from Cysteine	100	V	V	V	+	V
Casein hydrolysis	83.7	+	-	-	+	+
Starch hydrolysis	1.16	-	-	-	Nd	-
Sensitivity to erythromycin (15µg/disk)	0	-	-	-	-	+
Phosphatase	0	-	-	-	-	+
Utilization of keto-methyl glucoside	0	-	+	+	-	-
NaCl%5 tolerance	88.4	+	+	+	V	-
Acid production from:						
Lactose	73.75	+	+	+	-	+
Maltose	17	-	+	+	-	-
Trehalose	63.8	+	+	+	+	-
Sorbitol	20	-	-	-	-	-
Melibiose	100	+	+	-	-	+
Raffinose	100	+	+	V	-	+
D-Galactose	18	+	+	+	-	+
Inulin	18.3	-	-	+	-	Nd
Myo-Inositol	74.7	-	-	+	-	
Melezitose	14.6	-	-	-	Nd	-
Cellobiose	100	+	+	V	Nd	+
L-fucose	19.75	-	-	-	Nd	
L-Rhamnose	100	+	+	+	Nd	+
L-Arabinose	100	+	+	+	Nd	+
Utilization of:						
Citrate	96.4	+	+	-	+	+
Malonate	0	-	-	-	-	+
Glutamate	100	+	-	+	-	+
Tartrate	0	-	-	-	-	-
Growing at 37.0 °C	74.4	+	-	+	-	+
Growing at 40.0 °C	15.11	-	-	-	-	Nd

n, number of strains tested.
+, 80% or more strains positive; V, between 21-79% of strains positive; -, 80% or more strains negative; Nd, not determined.
[†], details of the *Dicheya chrysanthemi* and *Pectobacterium atrosepticum*, *Pectobacterium wasabiae*, *Pectobacterium betavascularum* and subspecies *Pectobacterium carotovorum* subsp. *carotovorum* based on Holt *et al* (32), *Gardan et al* (22) and Khayi *et al* (33).
[*], numbers correspond to the percentage of positive studied strains.

results were obtained from indole, phosphatase and urease production, oxidase reaction, malonate and keto-methyl glucoside utilization. All isolates produced H_2S from cysteine and did not possess phenylalanine deaminase activity. The positive result was obtained from gelatin liquefaction for 91.56% of strains, and from starch hydrolysis for only 1.16% of strains. About 88.4% of isolates could tolerate Nacl 5% and 11.5% reducing substances from sucrose. Acid production from lactose and maltose were positive for 73.75% and 17% of isolates respectively. All isolates could produce acid from melibiose and raffinose. On the other hand, none of the isolates utilized malonate and tartrate, although all utilized glutamate.

On fresh yeast extract dextrose calcium carbonate medium (YDC), the strains did not produce a characteristic dark blue insoluble pigment and they appeared non-fluorescent on King's B medium. All of the strains grew at 28°C; but only 74.4% and 15.11% grew at 37°C and 40°C respectively.

Regarding the results, the isolates belong to the genus *Pectobacterium.* However, atypical isolates were detected, which did not show typical biochemical reactions similar to standard isolates. Based on phenotypic tests,

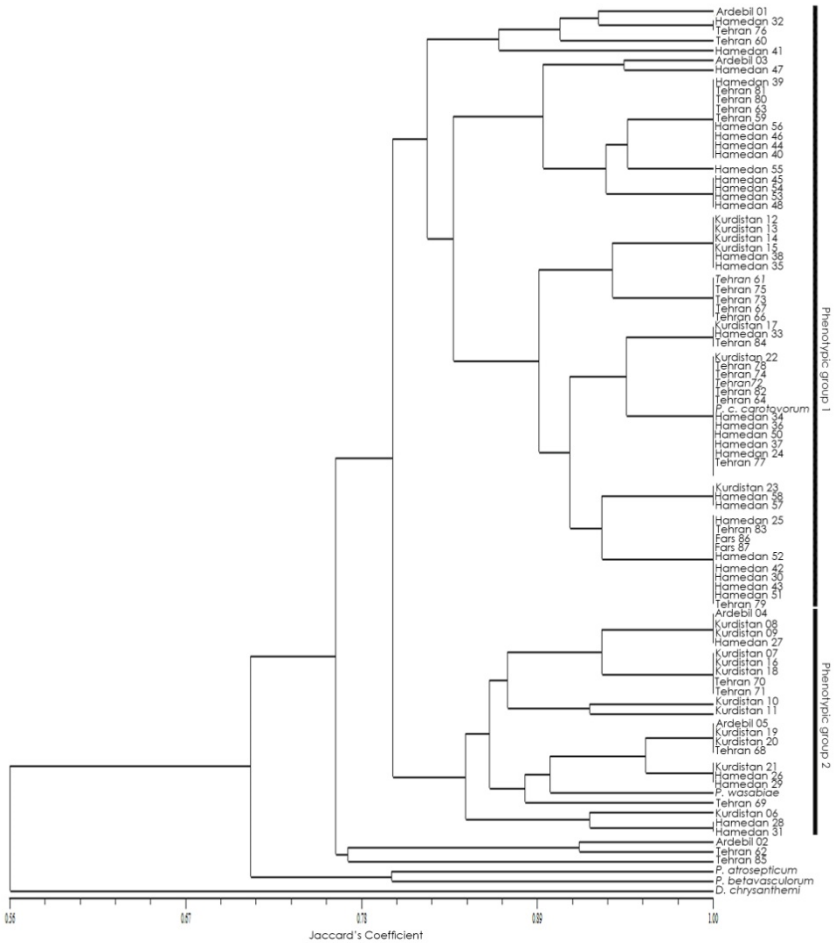

Figure 2: Dendrogram established from Jaccard's similarity coefficients between Iraninian potato soft rot isolates, using UPGMA method based on phenotypic traits. The studied isolates were divided into two main groups associated with *Pectobacterium wasabiae* and *P. carotovorum* subsp. *carotovorum*. *P. atrosepticum*, *P. betavasculorum* and *Dickeya chrysanthemi*, each were placed in groups that were separated from Iranian isolates.

the isolates were divided into two main groups (Figure 2). Detailed phenotypic characteristics are summarized in Table 2.

Genomic studies

BOX and ERIC primer sets gave reproducible genomic PCR profiles consisting of bands of approximately 150 to 6000bp. Amplification with BOX primer produced 23 distinct bands varying from 300 to 3500bp. ERIC1 PCR generated 27 distinct bands from 150 to 6000bp and ERIC2 PCR generated 21 fragments from 150 to 5000bp. The genomic fingerprints revealed high polymorphism among the strains.

A total of 29 reproducible bands consisting of seven informative bands for BOX primer, eight for ERIC1 and fourteen for ERIC2 primer were scored. In this study, ERIC2 primer was more discriminative than BOX and ERIC1 primer in differentiating the Iranian potato soft rot pathogens. The Bayesian analysis of Structure 2.3.4 assigned genotypes to three populations (K=3) (Figure 3).

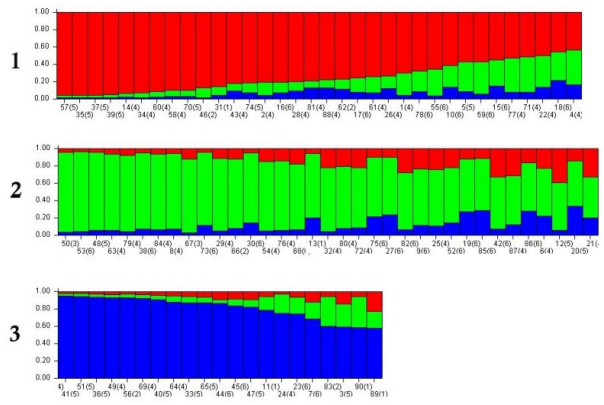

Figure 3: Genetic structure of Iranian SRP populations based on Bayesian clustering analysis [Structure software v2.3.4]; a STRUCTURE analysis showing three Iranian SRP isolate populations. The labels below the bar plots are the corresponding numbers for each strain. The numbers on the left represent the three genetic populations found among the isolates studied.

In order to confirm the results of the cluster analysis, the ΔK values were calculated by the Structure 2.3.4 software. In this study, based on the above criteria, k=3 is the best number of groups which are marked with separate colors in Figure 3.

Each individual is shown by one vertical bar plot (representing membership coefficient); the labels below the bar plots are the corresponding numbers for each strain. More than one color in each bar plot reveals the genetic complexity of that individual. In this case, the strain belongs to a group which has the largest color width of that cluster.

The network analysis indicated genetic diversity among the strains isolated from different geographical areas. The relationships among the Iranian SRP isolates were displayed as a phylogenetic network in Figure 4. Such diversity was so varied that made it impossible to categorize the strains into separate groups (Figure 4).

Analysis of molecular variance (AMOVA) revealed maximum genetic variation between Kurdistan-Fars isolates and minimum variation for Tehran-Ardebil isolates. After that, Kurdistan-Ardebil isolates had the least genetic diversity among the population. Evaluation of intra-population genetic variation of studied isolates showed that this index was the lowest for Fars isolates. In contrast, such index for Tehran, Kurdistan, Hamedan and Ardebil isolates was higher than Fars, and at the same time showed less variation between each of the named provinces. Lowest Nei's genetic distances (Ds) were found between Kurdistan-Ardebil isolates and highest for Kur-

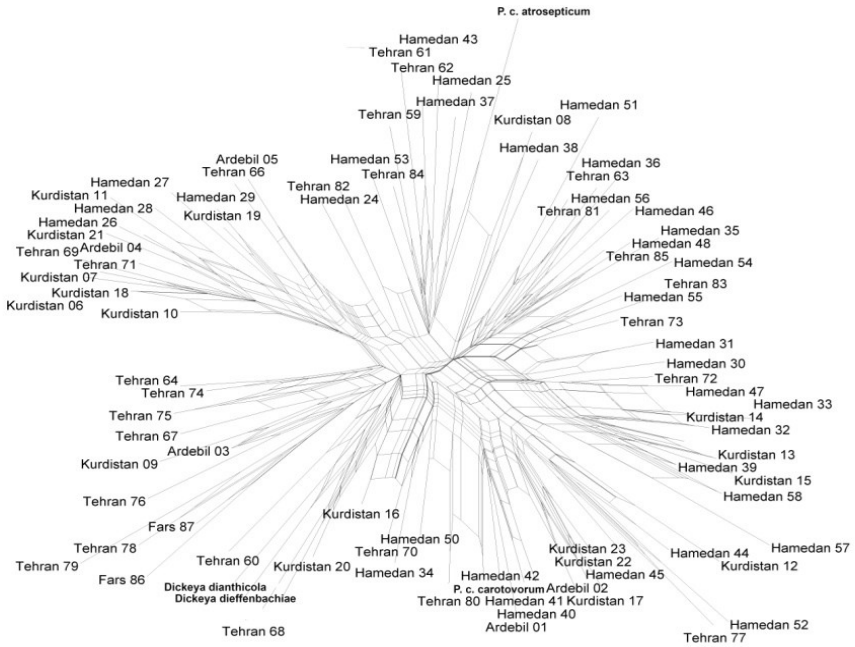

Figure 4: Molecular phylogenetic network among Iranian SRP strains in this study from combined data of REP, ERIC and BOX primer sets. Distance analysis was conducted by the Neighbor-Net algorithms and support values at each node were estimated by running 1000 bootstrap replicates.

distan-Fars (Figure 5).

According to FST test, maximum gene flow was observed between Ardebil-Tehran isolates and minimum for Ardebil-Fars (Figure 6).

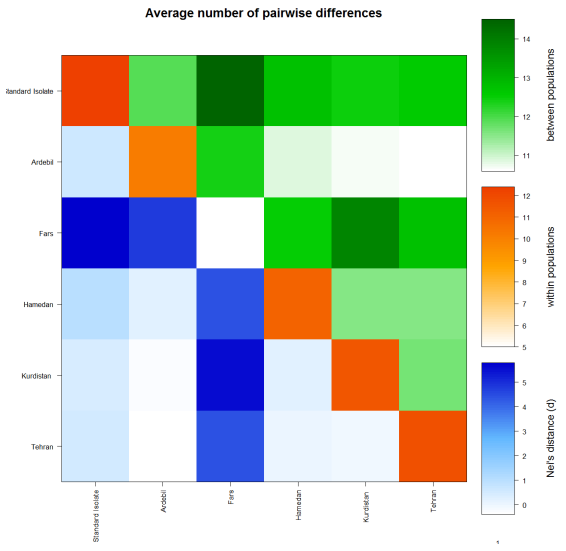

Figure 5: Nei's distance (d) and average number of pairwise differences within and between populations. The upper triangle of the matrix (green) denotes average pairwise differences between populations. The average number of pairwise differences within each population is denoted along the diagonal (orange). The lower triangle of the matrix (blue) shows differences between populations based on Nei's distance.

Discussion

Characterizing the diversity of potato soft rot

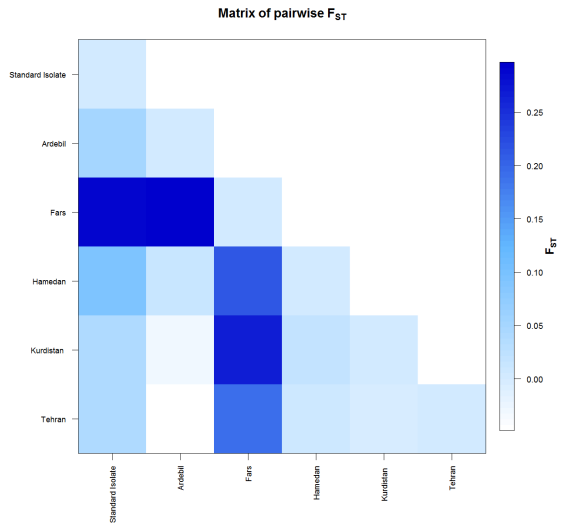

Figure 6: Population pairwise F_{ST} of comparisons of the five geographical populations and the standard isolates.

bacteria is the aim of the present study. The first report of potato soft rot disease in Iran was in 1986 (7), and after that there have been a few reports from regions cultivating potato (34, 35).

Although studies on bacterial phenotypic characteristics are time-consuming and in some cases with some intermediate responses, they are also very important in the study of plant pathogenic species. The phenotypic diversity is created through a complex network of interactions between the genotype and the surrounding environment. Creating a "genotype-phenotype" map requires accurate information from the phenotype to study these interactions (36). Therefore, in this study, the phenotypic diversity of isolates was evaluated.

Some isolates showed phenotypic characteristics that differed in biochemical and physiological characteristics of the isolates described in previous studies.

Also, the phenotypic diversity of the isolates was such that the isolates were placed in two major groups associated with *Pectobacterium wasabiae* and *Pectobacterium carotovorum* subsp. *carotovorum* (Figure 2). Group 1, which contained *P. carotovorum* subsp. *carotovorum* standard isolate, contained sixty isolates, of which 50% belonged to genetic population 1 and 48% to genetic population 2; only one isolate, Tehran66, belonged to genetic population 3. Also, group 2, in which the *Pectobacterium wasabiae* was located, contained twenty two isolates, 77.3% of them belonging to the genetic population 3.

Pectobacterium atrosepticum, *P. betavasculorum* and *Dickeya chrysanthemi*, each were placed in groups that were separated from Iranian isolates. Three isolates, Ardebil 02, Tehran 62 and Tehran 85 were not included in any group containing defined strains.

In phenotypic studies on Iranian SRP, we observed diversity among studied isolates. Even some of the isolates showed different response to biochemical and physiological tests, and hence their phenotypic characteristics did not match any of the *Pectobacterium* and *Dickeya* species and subspecies standard and type isolates. Similar observations have been reported for SRP by Duarte *et al* (37), Costa *et al* (38) and Vimal & Anuradha (39).

The results of phenotypic characteristics could not definitely identify the studied isolates below the genus level, and numerous atypical isolates are detected which do not show typical biochemical reactions; the same was previously reported by Yap *et al* (40) and Merwe *et al* (16). Czajkowski *et al* (6) mentioned that biochemical and physiological tests can give rise to ambiguous and equivocal results, and due to high strain variation, there are no reliable phenotypic tests available for distinguishing *Pectobacterium* species and subspecies.

Contrary to the statements of Czajkowski *et al* (6) that closely related species/subspecies maybe missed, as biochemical methods are based on the restricted number of key tests. However, in this study a phenotypic variation was observed among similar isolates, suggesting significant phenotypic variation among Iranian SRP isolates.

For molecular analysis, we performed repetitive extragenic palindromic PCR (rep-PCR) analysis, which generated different DNA fingerprints for isolates. This method

generates patterns and allows phylogenetic classification of SRP from genus down to strain level, and has been used extensively for the classification of *Pectobacterium* and *Dickeya* species strains (6).

Other studies showed success in using rep-PCR technique for molecular typing of *Pectobacterium carotovorum* subsp. *carotovorum* as well as other bacteria such as *Escherichia coli*, *Exserohilum turcicum*, *Vibrio parahaemolyticus*, and *Xanthomonas* (41).

SplitsTree network shows that there is a vast heterogeneity among studied isolates. The STRUCTURE software classified these strains into three genetic populations. No correlation between genomic fingerprints, geographic areas and potato cultivars was observed. Similar observation has been reported by Terta *et al* (14) in Morocco and Baghaee *et al* (42) in Iran. Although it is worth noting that Tavasoli *et al* (11) and Dehaghin and Shams-Bakhsh (13) observed correlation between rep-PCR genomic profiles and the geographical origins. Due to their findings SRP collected from the same regions tend to cluster together.

The maximum and minimum gene flow was observed between Ardebil-Tehran, the two near provinces and Ardebil-Fars, the two faraway provinces, respectively. Results from the FST test confirmed this observation. Interestingly, there was significant correlation between FST and geographic distance.

Based on pairwise Nei Distance values, the low distance was observed between Ardebil-Tehran populations and the high distance for Kurdistan -Fars populations. In the next step, the highest genetic distance was observed between isolates of Ardebil, Hamedan and Tehran with Fars isolates.

Estimating population average pairwise differences demonstrated the least genetic variation among the populations of Tehran-Ardebil, Kurdistan-Ardebil and Hamedan-Ardebil, respectively. Vice versa, the most genetic variation was among Kurdistan-Fars populations and, in some degree, between the isolates of Ardebil, Hamedan and Tehran with Fars populations.

According to these results, it is possible that SRP has been introduced to new territories in the neighboring provinces by seed trade, and the seed tuber could be the main source of soft rot disease contamination in potato fields in the studied regions in Iran.

In terms of genetic variation within the populations, Fars isolates were the least variant, which could be due to the number of samples studied in this region. In contrast, the isolates from Tehran, Kurdistan, Hamedan and Ardebil had similar genetic differences within populations.

Motyka *et al* (3) mentioned that some factors such as adequate set of the reference strains affect the discriminative powers of the rep-PCR technique. Using a higher number of reference strains could improve the evaluation of the diversity of the isolates. Therefore, we suggest the use of more reference strains in future studies.

SRP are among the top ten most important bacterial pathogens in agricultural sector (5). Certainly, correct taxonomic and diagnostic identification of plant pathogens has a great role in disease control and in supporting food security. Dees *et al* (43) introduced the novel species, *Pectobacterium polaris*, and

emphasized the need for re-evaluation and re-classification of *Pectobacterium*. It is suggested that more comprehensive studies be carried out on Iranian SRP isolates in order to establish a complete understanding of the tangled relationship between genotypes, phenotypes, and taxonomy.

Iran, as the largest producer of potato in west Asia, has a significant diversity of SRP. Hence, it is necessary to have accurate, specific, rapid and cost-effective screening methods for identification of isolates.

Accurate identification of genetic structure of these pathogens will facilitate the potato breeding programs and epidemiological studies in local, national, regional and international levels.

Conclusion

This study suggest that potato soft rot phytopathogen strains have spread throughout the potato fields, and are common in five different growing regions in northwest, central and south part of Iran. The results indicate that there is an apparent diversity between isolates, and strains could be divided into three groups. In each group, a number of studied isolates was found to be grouped with standard isolates, but with some variations in phenotypic traits.

This suggests the possibility of the existence of atypical strains among potato soft rot isolates in Iran. Meanwhile, it is possible that seed trade may introduce soft rot disease to new territories and the seed tuber could be the main source of soft rot disease contamination in potato fields in the studied regions in Iran.

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